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Inherited mutations in the breast and ovarian cancer susceptibility gene BRCA1 are associated with a high risk for developing breast and ovarian cancers. While numerous studies link BRCA1 to transcriptional regulation, DNA repair, apoptosis and growth/tumor suppression, the overexpression of the ErbB-2 family of receptor tyronsine kinases has been linked to the development of non-familial or sporadic breast cancer. Activation of HRG signaling, mediated by the binding of HRG to ErbB receptors, has been implicated in the development of aggressive phenotype in breast cancer cells. The mechanisms through which HRG regulates the progression of breast cancer cells to a more invasive or motile phenotype are currently unknown.

Our specific aims are:

1) To determine the effect of ECM/integrins on BRCA1 expression, phosphorylation and nuclear translocation.
2) To generate inducible stable transfected T47D clones that overexpress BRCA1 protein, in order to characterize its biological functions in breast cancer cells.
3) To determine the effect of PTEN on BRCA1 phosphorylation.

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Title: Biological function of BRCA1 and its regulation by the Extracellular matrix and PTEN in breast cancer

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Inherited mutations in the breast and ovarian cancer susceptibility gene BRCA1 are associated with a high risk for developing breast and ovarian cancers. While numerous studies link BRCA1 to transcriptional regulation, DNA repair, apoptosis and growth/tumor suppression, the overexpression of the ErbB-2 family of receptor tyrosine kinases has been linked to the development of non-familial or sporadic breast cancer. Activation of HRG signaling, mediated by the binding of HRG to ErbB receptors, has been implicated in the development of aggressive phenotype in breast cancer cells. The mechanisms through which HRG regulates the progression of breast cancer cells to a more invasive or motile phenotype are currently unknown.

The multiple functions of the BRCA1 protein include growth/tumor suppression, induction of apoptosis, cell cycle regulated expression, DNA repair and the maintenance of genomic stability. Despite several lines of evidence suggesting that serine phosphorylation of BRCA1 during cell cycle progression and in response to DNA damaging agents may affect its function, the signaling pathway(s), which leads to the phosphorylation of BRCA1 has not been described.

HRGs are a family of polypeptide growth factors derived from an alternatively spliced gene. They bind tyrosine kinases ErbB-3 and ErbB-4 receptors, inducing their heterodimerization with ErbB-2 leading to receptor tyrosine phosphorylation and activation of downstream signal transduction. Activation of HRG signaling has been implicated in the development of aggressive phenotype in breast cancer cells.

The extracellular matrix (ECM) is composed of several proteins such as fibronectin, collagens and laminin. ECM plays an important role in morphogenesis, tissue repair and regeneration, as well as metastasis. An altered interaction between cells and the surrounding ECM is a common feature of a wide variety of tumors. ECM is capable of activating the PI-3K/AKT pathways through integrins.

While BRCA1 has been shown to act as a growth suppressor, activation of the ErbB-2 receptor by HRG has been shown to promote cell growth in breast epithelial cells. Our recent study indicates a link between BRCA1 and HRG function. BRCA1 phosphorylation is mediated by the PI-3K/AKT pathway upon HRG stimulation of breast cancer cells. These results lead us to hypothesize that: (1) BRCA1 might play a role not only in the onset of familial breast cancer but also in sporadic breast cancer; (2) Inhibition of normal BRCA1 function by HRG could contribute to the dysregulated cell growth associated with sporadic breast cancer; and (3) BRCA1 expression and phosphorylation might be regulated by the ECM.

Our specific aims are:

1) To determine the effect of ECM/integrins on BRCA1 expression, phosphorylation and nuclear translocation.
2) To generate inducible stable transfected T47D clones that overexpress BRCA1 protein, in order to characterize its biological functions in breast cancer cells.
3) To determine the effect of PTEN on BRCA1 phosphorylation

HRG inhibits the growth suppressive effect of BRCA1:

The finding that activation of AKT triggers BRCA1 phosphorylation suggests that HRG, a growth factor for T47D cells, might increase cell growth by phosphorylating and subsequently negatively regulating the growth suppressive activity of BRCA1. To test this possibility, we took advantage of a well-established growth analysis system (17). By using green fluorescent protein (GFP) to mark specific transfected cells, new DNA synthesis was determined in transiently transfected breast cancer cells. The percentage of GFP-expressing cells undergoing DNA synthesis after 2 days of transfection was determined by the ability of cells to incorporate BrdU. As shown in Fig. 1A, BrdU incorporation was reduced by about 50% in the BRCA1-transfected cells relative to cells transfected with an empty vector. However, in the presence of HRG, ectopic expression of

BRCA1 led to only about a 10% decrease in BrdU incorporation in the GFP-expressing cells. In cells co-expressing BRCA1 and either K227E p110 or myr-AKT, no significant decrease in BrdU incorporation was observed. The expression of transfected plasmids was confirmed by Western blot analysis (data not shown). Negative regulation of the growth suppressive function of BRCA1 by HRG and by its constitutively active signal transduction components PI-3K and AKT suggests that phosphorylation of BRCA1 by AKT can interfere with the biological activity of BRCA1.

It has previously been shown that BRCA1 can increase the expression of the cyclin dependent kinase inhibitor $p21^{WAF1/CIP1}$ by transactivating its promoter, which might play an important role in BRCA1-mediated growth inhibition in cancer cells. To test whether phosphorylation of BRCA1 by PI-3K/AKT negatively regulates the BRCA1 function that transactivates the $p21^{WAF1/CIP1}$ promoter, T47D cells were co-transfected with the $^{WAF1/CIP1}$-luciferase plasmid (pWWP-Luc) together with either HA-BRCA1 alone or with the myr-AKT or K227E p110 expression plamid. Cells were untreated or treated with HRG, and luciferase activity was determined 24 h after transfection. Consistent with the previous report (17-19), BRCA1 increased the luciferase activity about 3-fold, as compared to cells transfected with vector alone (Fig. 1B) Furthermore, HRG treatment as well as myr-AKT and K227E p110 significantly inhibited the BRCA1-mediated activation of the $p21^{WAF1/CIP1}$ promoter. These results strongly suggest that the HRG-induced phosphorylation of BRCA1 by AKT negatively regulates the $p21^{WAF1/CIP1}$ transactivating activity of BRCA1. These results also suggest a mechanism by which HRG and its signal transduction components block the growth suppressive activity of BRCA1 in breast cancer cells.

Effect of ECM/integrins on BRCA1 expression and phosphorylation.

Treatment with heregulin caused a transient phosphorylation of BRCA1, peaking between 0.5-1 h in T47D breast cancer cells (Figure 2A). When these cells were grown on laminin (LAM), the level of BRCA1 phosphorylation was significantly increased in the presence of HRG (Figure 2B). However, this phosphorylation was much lower in cells seeded onto poly-L-lysine (POL) or maintained in suspension (SUS), suggesting integrin involvement in this process. T47D cells indeed do express β_1, β_4 and α_6 integrins (data not shown).

We sought to determine the effect of extracellular matrix on BRCA1 expression at both the protein and mRNA levels after a time course of heregulin treatment (Figure 3). Our results showed a decrease in BRCA1 mRNA level at 4 h, followed by a gradual increase in the amount of BRCA1 mRNA (peaking at 12-18 h). We then analyzed the effect of several matrices on BRCA1 mRNA level at five (0, 4, 14, 24, and 48 h) characteristic time points during heregulin treatment. When T47D cells were seeded on Plastic (PL), LAM or Collagen (COL), a similar pattern of BRCA1 expression was observed in cells grown on all three matrices (Figure 4): a relatively high basal level of BRCA1 expression, very low at 4 h after heregulin treatment, very high mRNA level at 14 h and a gradual decrease at the following time points such as 24 and 48 h (Figure 4). However, there was a significant decrease at in the basal level and at the 4 h-time point of BRCA1 mRNA expression in cells grown on laminin and collagen type IV. Likewise, there was a strong decrease in the protein expression of nontreated cells (Figure 5). Interestingly, the level of BRCA1 protein was slightly increased at 14 h after heregulin treatment only in cells seeded on PL. Cell pretreatment with N-benzyloxycarbonyl-Ile-Glu (*O*-t-butyl)-Ala-leucinal (PSI), a proteasome inhibitor, restored the amount of BRCA1 protein at the basal level suggesting the involvement of proteasome-dependent degradation (Figure. 5).

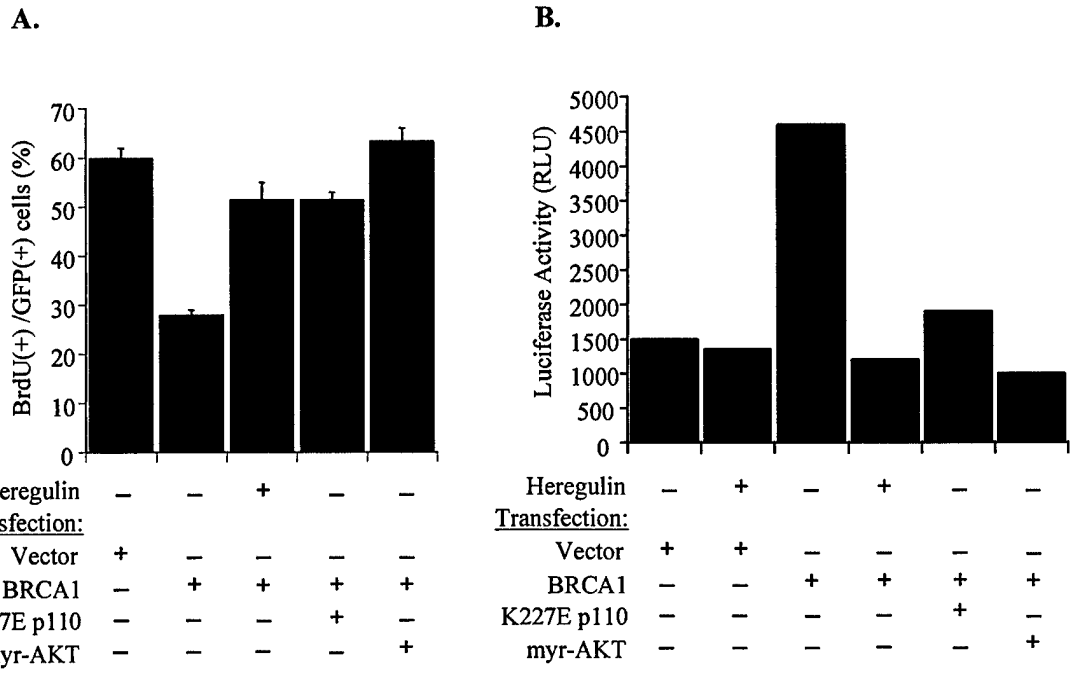

Figure 1: The growth inhibitory effect and $p21^{WAF1/CIP1}$ transactivating activity of BRCA1 in breast cancer cells are blocked by HRG and its constitutively active signaling components, PI-3K and AKT.

(A) The green fluorescent protein (GFP) expression vector was co-transfected into T47D cells either with empty vector or with the BRCA1 vector alone or together with either K227E p110 or myr-AKT expression plasmid in a 1:5 ratio. After transfection, cells were untreated (-) or treated (+) with heregulin (HRG) for 2 days and pulsed with BrdU for 1 h. The percentage of BrdU incorporation into the GFP-expressing cells was determined from two independent experiments by analyzing 100 GFP(+) cells in each experiment.

(B) T47D cells were co-transfected with $p21^{WAF1/CIP1}$-luciferase plasmid (pWWP-Luc) and either with BRCA1 alone or together with the K227E p110 or myr-AKT plasmid. ϒ-galactosidase was used as an internal control to monitor transfection efficiency. Cells were untreated (-) or treated (+) with heregulin, and activity was measured 24 h. later.

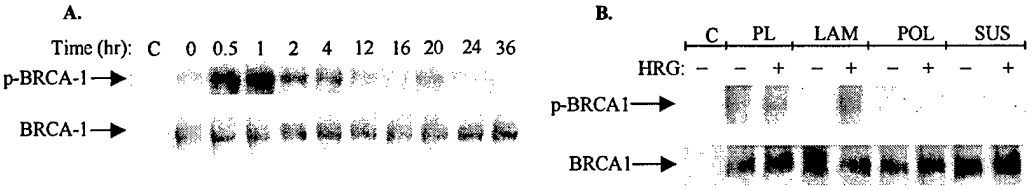

Figure 2. ECM affects heregulin-dependent BRCA1 phosphorylation:

A) T47D cells were seeded on plastic Petri dishes and starved for 48 h. Four hours prior to stimulation with heregulin, cells were loaded with $[^{32}P]H_3PO_4$. Total cell lysates were then collected at the indicated times and immunoprecipitated with anti-BRCA1 antibody (C-20).

B) In addition to being seeded onto plastic, cells were seeded onto plates coated with laminin (LAM), poly-L-lysine (POL) or maintained in suspension (SUS) after the starvation and labeling period. Cells were than treated for 30 min and processed the same as those seeded on plastic. Immunoprecipitates from A and B were run on SDS-PAGE and the level of phosphorylation was detected by autoradiography (upper panels). Portions of the immunoprecipitates were saved and run on separated gels for Western blotting (lower panels), and probed with another anti-BRCA1 antibody (BRIH-945.2). "C" indicates the sample containing cell lysates plus protein-G agarose (without antibody). The results are representative of two independent experiments.

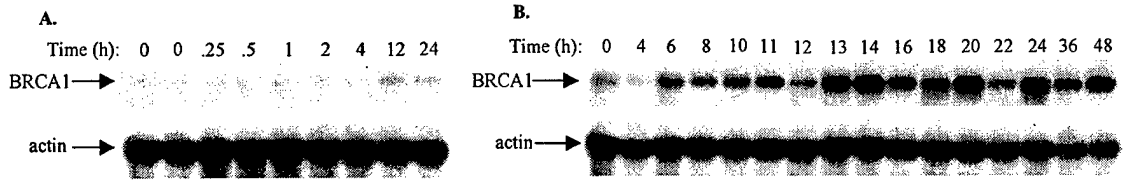

Figure 3. Heregulin affects BRCA1 mRNA level:
T47D cells were treated with heregulin for the indicated times and total mRNA was isolated by using a Qiagen kit. Northern blots were probed with a specific probe for BRCA1 (upper panels), and followed by probing for actin (lower panels) after stripping the membrane.

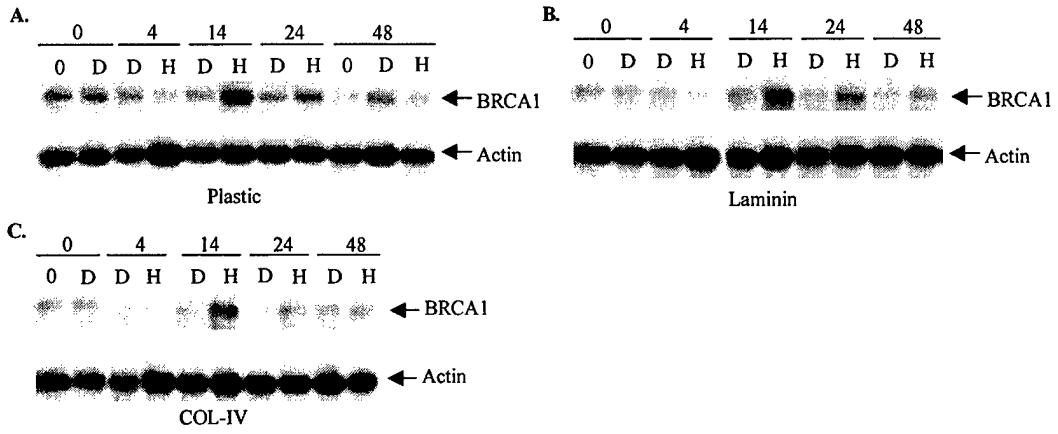

Figure 4. Effect of ECM on BRCA1 expression:
T47D cells were seeded on plastic (A), laminin (B), or on collagen type-IV (COL-IV), (C), starved as in Figure 1 and stimulated with heregulin for the indicated times. Northern blotting was performed the same way as in Figure 3. Autoradiograms are representative of two independent experiments.

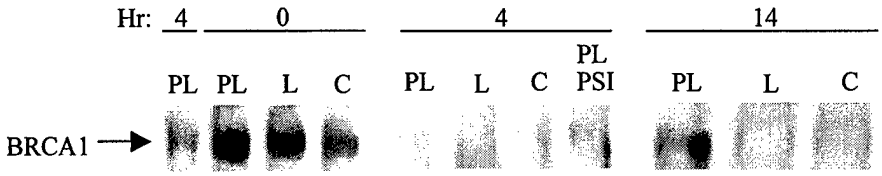

Figure 5. Effect of ECM on BRCA1 protein expression in T47D cells:
Cells seeded on plastic (PL), laminin (L), or on collagen type-IV (C) were starved and stimulated with heregulin (20 nM) for the indicated times (h). Equal amounts of total cell lysates were separated by SDS-PAGE and subjected to Western blotting. Membranes were probed either with anti-BRCA1 antibody (BRIH-945.2, upper panels). Blots are representative of two independent experiments.

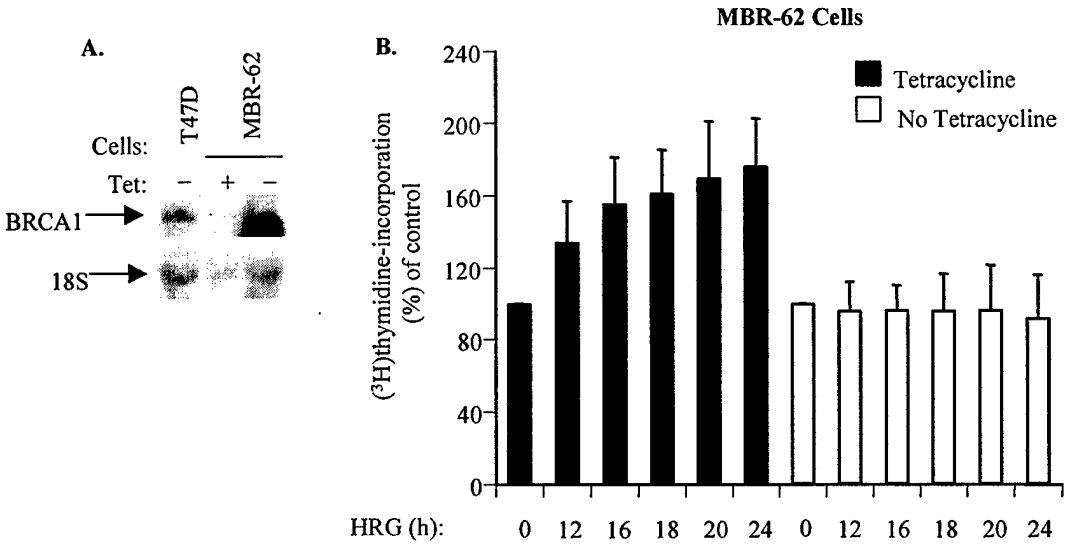

Figure 6. BRCA1 affects heregulin-dependent DNA synthesis in MBR62 breast cancer cells:
MBR62 breast cancer cells possess a tetracycline sensitive promoter of the BRCA1 protein. These cells were seeded onto plastic Petri dishes and grown in the absence (Tet-) or presence (Tet +) of tetracycline (2 μg/ml). A) Cells were lysed and processed for Northern blotting. The membranes were probed with BRCA1 (upper panel) or 18S probes (lower panel). Cells grown in the absence (white bars, no tetracycline) or the presence of tetracycline (black bars) were starved and stimulated with heregulin for the indicated times. DNA synthesis was determined by the amount of [^{3}H]thymidine incorporation after cell treatment with heregulin. The experiment was repeated two times.

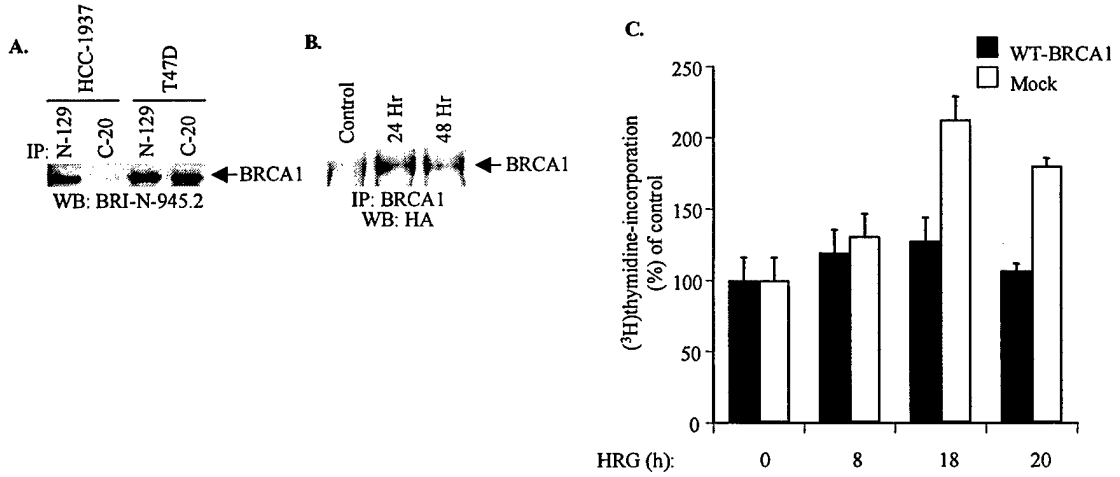

Figure 7. BRCA1 affects heregulin-dependent DNA synthesis in HCC-1937 cells:

A) HCC-1937 and T47D cells grown on plastic Petri dishes were lysed in RIPA buffer, and total cell lysates were subjected to immunoprecipitation either with anti BRCA1 antibodies: BRIN-129 (N-129) or C-20. The immunoprecipitates were run on SDS-PAGE, subjected to Western blotting, and then membranes were probed with another anti-BRCA1 monoclonal antibody: BRIH-945.2.

B) Plasmid DNA containing wild-type BRCA1 with a hemagglutinin (HA) tag was overexpressed in HCC-1937 cells for 24 or 48 h. Cell lysates containing empty vector (control) or the BRCA1 construct (24 h and 48 h) were immunoprecipitated with anti-BRCA1 antibody and subjected to Western blotting. Membranes were probed with an anti-HA antibody, followed by ECL autoradiography.

HCC-1937 cells overexpressing empty vector (Mock, white bars), or vector containing DNA encoding BRCA1 protein (WT-BRCA1, black bars), were starved and treated with heregulin (20 nM) for the indicated times. DNA synthesis was determined. Values are the mean +/- SD and are expressed as a percent of the control, nontreated cells (taken as 100%).

Effects of BRCA1 on HRG-dependent proliferation

Stable inducible transfected MBR62 cells that overexpress BRCA1 protein were kindly obtained from Dr. Dan Haber (18) (MGH, Boston). In the presence of tetracycline (tet), there was no expression of BRCA1 while in the absence of tet, BRCA1 protein is expressed (Figure 6A). With low expression of BRCA1, MBR62 cells responded to heregulin with a strong increase in [^{3}H]thymidine incorporation over 24 h (Figure 6B). However, when tetracycline was removed from the growth medium, cell response to heregulin was abolished, suggesting that increased expression of BRCA1 down-regulated the heregulin-dependent mitogenic response. In addition, we used HCC-1937 cells that contained a truncated C-terminal, and therefore non-functional BRCA1 protein (Figure 7A). When starved HCC-1937 cells were treated with heregulin, a strong increase in DNA synthesis was observed, peaking at 18 h. This increase in DNA synthesis was strongly inhibited by the presence of overexpressed wild type BRCA1 protein (Figure 7B and 7C).

CONCLUSIONS

1) Extracellular matrix effected a heregulin-dependent phosphorylation of BRCA1 in T47D breast cancer cells, since this was phosphorylation was greatly increased in cells seeded on laminin. In contrast, the BRCA1 phosphorylation in cells grown on poly-L-lysine or in cells maintained in suspension was significantly lower than that in cells seeded on plastic. This process is most likely regulated by β_4 rather than β_1 integrin, since cells seeded on laminin showed increased tyrosine phosphorylation of the ErbB-2 receptor and increased PI3 kinase activity while these signals were only modestly increased in cells grown on fibronectin (data not shown).

2) There was a strong decrease in both mRNA and protein level of BRCA1 4 h after treatment with heregulin as compared to the basal level of the nontreated cells. ECM also affected the expression of BRCA1 at both the mRNA and protein levels. There was a significant decrease at the basal level of BRCA1 mRNA expression in cells seeded on laminin or on collagen type IV as compared to that in cells seeded on plastic. Pretreatment with the proteasome specific inhibitor, PSI, restored BRCA1 expression in these cells.

3) BRCA1 suppressed the heregulin-dependent DNA synthesis in breast cancer cells and expression of wild-type BRCA1 abolished the mitogenic response in both MBR62 and HCC-1937 cells.

These studies should provide insights into the molecular functions of BRCA1 and determine whether inhibition of BRCA1 function by HRG could contribute to the dysregulated cell growth associated with breast cancer. The novelty of this project is the data of HRG and ECM modulating BRCA1 expression and phosphorylation, as well as the involvement of proteasome-dependent degradation of BRCA1 upon HRG and ECM treatment. The concept that an inhibitory effect mediated by BRCA1 could be down-regulated by growth-promoting signals is highly movel in the field of BRCA1.

Published Studies on This Work:

1 Price DJ, Miralem T, Jiang S, Steinberg R, Avraham H. Role of VEGF in the stimulation of cellular invasion and signaling of breast cancer cells. Cell Growth and Differentiation 2001; 12:129-135.

2 Price, D.J., Avraham, S., Feuerstein J., Fu, Y., and Avraham, H. The Invasive phenotype in HMT-3522 cells requires increased EGF Receptor signaling through both PI-3kinase and E-RK 1, 2 pathways. Cell Communication and Adhesion. 9, 2002, 87-102.

3 Miralem T, Steinberg R, Price D, Avraham H. VEGF165 requires extracellular matrix components to induce mitogenic effects and migratory response in breast cancer cells. Oncogene. 2001; 20:5511-5524.

4 Miralem, T. and H. Avraham, Extracellular matrix enhances HRG-dependent phosphorylation of BRCA1 and suppresses its expression through C-terminus in breast cancer cells. Mol Cell Biol. 2003, 23:579-593.

Tihomir Miralem, Ph.D. was the only person paid from this study.